Vol. 117, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1983 Pages 541-548

EVIDENCE FOR THE METABOLIC FORMATION OF A VICINAL DIHYDRODIOL-EPOXIDE FROM THE POTENT MUTAGEN 1-NITROBENZO(A)PYRENE

MING W. CHOU AND PETER P. FU

National Center for Toxicological Research, Jefferson, AR 72079

Received November 3, 1983

Summary: Metabolism of 1-nitrobenzo(a)pyrene (1-nitro-BaP) by rat liver microsomes yielded 1-nitro-BaP trans-7,8-dihydrodiol, 1-nitro-BaP trans-9,10-dihydrodiol and 1-nitro-BaP 7,8,9,10-tetrahydrotetrol. Formation of these metabolites suggests that a vicinal 7,8,9,10-dihydrodiol-epoxide is a metabolite of 1-nitro-BaP.

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) have recently been identified as a new class of potent mutagenic and carcinogenic environmental pollutants existing in fly ash, diesel emissions, photocopier toners, cigarette smoke and soils (1-5). Since many nitro-PAHs are widespread in the environment and are carcinogenic in experimental animals (5,6) a major concern now is the possible hazard of these compounds to human health. Published results indicated that both reduction of the nitro substituent of nitro-PAHs (7,8) and ring oxidation (9-12) can be involved in the metabolic activation. However, it is not known if nitro-PAHs can be metabolized to vicinal diol-epoxides, the activated form of PAHs. 1-Nitro-BaP, together with the 3- and 6-nitro-BaP isomers, has been found to be formed in model atmospheres containing trace quantities of BaP, nitrogen oxide and nitric acid (13). We now report that the in vitro aerobic metabolism of 1-nitro-BaP, a potent bacterial mutagen both in the presence and absence of mammalian liver homogenate activation enzymes (14), yields trans-7,8- and 9,10-dihydrodiols and 7,8,9,10-tetrahydrotetrol. These results suggest that a vicinal dihydrodiol-epoxide can be a metabolite of a nitro-PAH.

Abbreviations: Nitro-PAH, nitro-polycyclic aromatic hydrocarbon; BaP, benzo(a)-pyrene; 1-nitro-BaP trans-7,8-dihydrodiol, trans-7,8-dihydroxy-1-nitro-7,8-dihydro-BaP; 1-nitro-BaP 7,8,9,10-tetrahydrotetrol, 7,8,9,10-tetrahydroxy-1-nitro-7,8,9,10-tetrahydro-BaP; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

0006-291X/83 $1.50

MATERIALS AND METHODS

Synthesis of 1-Nitrobenzo(a)pyrene: 9,10-Dihydrobenzo(a)pyren-7(8H)-one (Aldrich Chem. Co.) was reduced to 7,8,9,10-tetrahydro-BaP via Wolff-Kishner reduction (15). Nitration of 7,8,9,10-tetrahydro-BaP with sodium nitrate in trifluoroacetic acid and trifluoroacetic anhydride at ambient temperature under argon gave a mixture of 1-, 3- and 6-nitro-7,8,9,10-tetrahydro-BaP in good yield (16). The reaction mixture was separated by HPLC employing a DuPont Zorbax SIL column (9.4 x 250 mm) and eluting with 2% tetrahydrofuran in hexane in a flow rate of 3 ml/min. The retention time of 1-nitro-7,8,9,10-tetrahydro-BaP was 18.2 min. Mass spectral analysis indicated molecular ion at m/z 301. Dehydrogenation of 1-nitro-7,8,9,10-tetrahydro-BaP with dichlorodicyanobenzoquinone (17) gave 1-nitro-BaP which was purified by chromatography on a Florisil column with benzene as the eluting solvent; mp 250-250.5°, m/z of M^+ at 297; 500 MHz proton NMR (acetone-d_6) δ 7.94 (dd,1,H-8), 8.02 (dd,1,H-9), 8.19 (d,1,H-4), 8.36 (d,1,H-3), 8.39 (d,1,H-5), 8.49 (d,1,H-7), 8.78 (d,1,H-2), 8.94 (s,1,H-6), 9.12 (d,1,H-12), 9.32 (d,1,H-10), 9.57 (d,1,H-11); $J_{2,3}$ = 8.3, $J_{4,5}$ = 9.2, $J_{11,12}$ = 9.6 Hz.

In Vitro Incubations: Male Sprague-Dawley rats (80-100 g), obtained from our breeding colony, received intraperitoneal injections of 3-methylcholanthrene (25 mg/kg body weight) on 3 consecutive days before sacrifice. Liver microsomes were prepared as previously described (18). Incubation mixtures contained 50 mmol Tris-HCl, pH 7.5, 3 mmol $MgCl_2$, 1 mmol $NADP^+$, 2 mmol glucose-6-phosphate, 100 units glucose-6-phosphate dehydrogenase, 1 g microsomal protein and 40 µmol 1-nitro-BaP (dissolved in 40 ml acetone) in a total incubation volume of 1 l. Incubations were conducted aerobically with shaking for 60 min at 37° and then quenched by the addition of 1 l acetone. The metabolites and residual substrate were partitioned into 2 l ethyl acetate, and the organic phase was dried with anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was dissolved in 1 ml methanol for analysis by HPLC.

Separation and Characterization of Metabolites: Reversed-phase HPLC was performed with a Beckman system consisting of two model 100A pumps, a model 210 injector, a model 420 solvent programmer and a Waters Associates model 440 absorbance (254 nm) detector. Metabolites were separated by using a DuPont Zorbax ODS column (9.4 mm x 25 cm) and eluting with a 20-min linear gradient of 50-100% methanol in water at a flow rate of 3 ml/min.

Uv-visible spectra were obtained with a Beckman model 25 spectrometer. Mass spectra were recorded with a Finnigan model 4000 system. The samples were introduced with a solid probe and ionized at 70 eV with an ionizer temperature of 250°. ^1H NMR spectra were obtained with a Bruker WM 500 spectrometer.

RESULTS AND DISCUSSION

The HPLC profile of the ethyl acetate extractable metabolites obtained from incubation of 1-nitro-BaP with rat liver microsomes is shown in Figure 1. The chromatographic peak 4 contained the recovered substrate 1-nitro-BaP. The uv-visible absorption spectra of the metabolites contained in chromatographic peaks 2 and 3 are shown in Fig. 2A. Both of these metabolites had similar mass spectra (Fig. 3) with a molecular ion at m/z 331 and a major fragment at m/z 267 due to loss of a water molecule and NO_2. The structures of these metabolites were determined by analysis of their high resolution 500 MHz proton NMR

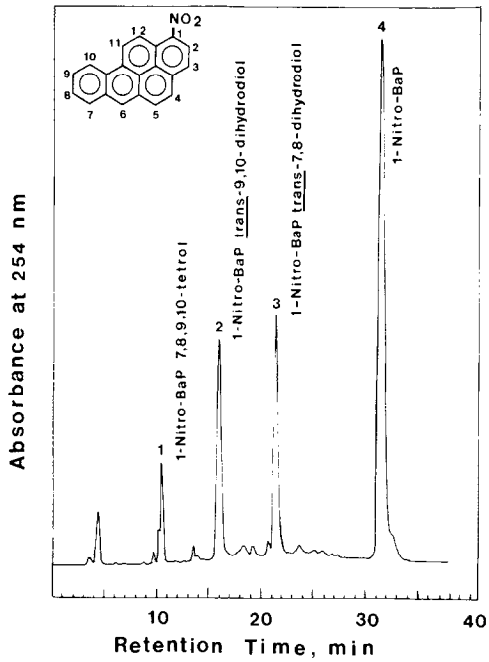

Fig. 1. Reversed-phase HPLC profile of ethyl acetate extractable metabolites obtained from incubation of 1-nitro-BaP with liver microsomes from 3-methylcholanthrene-treated male Sprague-Dawley rats. The chromatographic conditions are described in Materials and Methods.

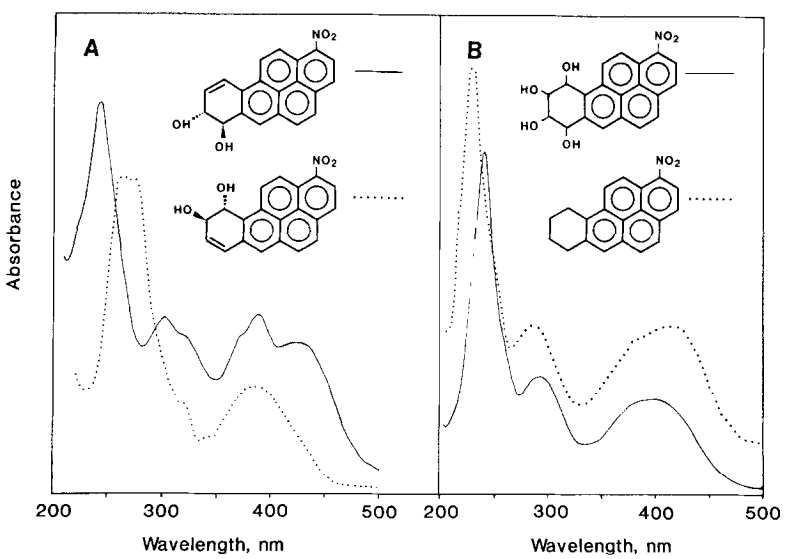

Fig. 2. Ultraviolet-visible absorption spectra (methanol) of (A) the metabolites identified as 1-nitro-BaP trans-7,8-dihydrodiol (contained in peak 3 of Fig. 1) (——) and 1-nitro-BaP-9,10-dihydrodiol (contained in peak 2 of Fig. 1) (·····) and (B) the metabolite contained in peak 1 of Fig. 1 identified as 1-nitro-BaP 7,8,8,10-tetrahydrotetrol (——) and the synthetic compound 7,8,9,10-tetrahydro-1-nitro-BaP (·····).

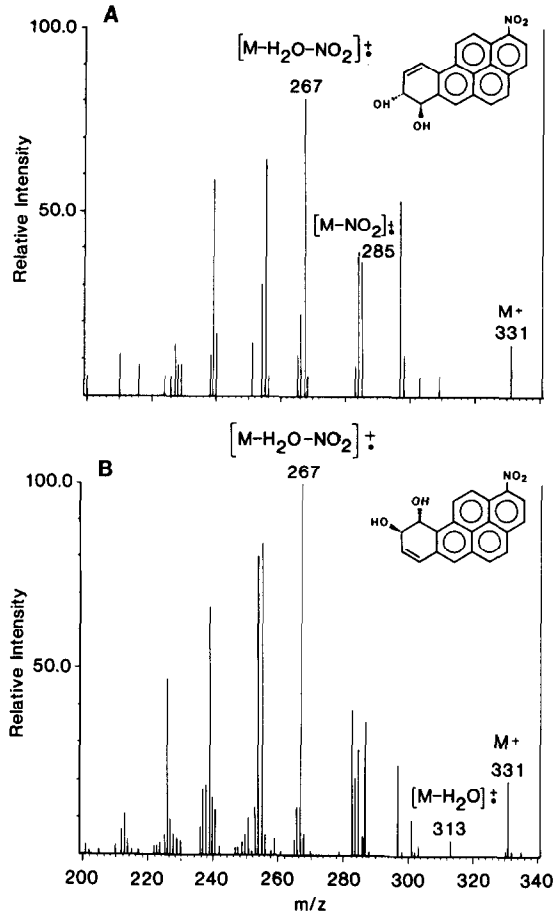

Fig. 3. Mass spectra of the metabolites identified as (A) 1-nitro-BaP trans-7,8,-dihydrodiol and (B) 1-nitro-BaP trans-9,10-dihydrodiol.

spectra (Fig. 4). The NMR resonance assignments were determined both by comparison to BaP trans-7,8- and 9,10-dihydrodiols (19) and by extensive homonuclear decoupling experiments. Based on the mass and NMR spectral analysis, the metabolites contained in peaks 2 and 3 were identified as 1-nitro-BaP trans-9,10-dihydrodiol and 1-nitro-BaP trans-7,8-dihydrodiol, respectively. The proton NMR assignmens are as follows: 1-Nitro-BaP trans-7,8-dihydrodiol (acetone-d_6 with trace D_2O): 4.67 (dt,1, H_8), 5.16 (d,1,H_7), 6.44 (dd,1,H_9), 7.65 (dd, 1,H_{10}), 8.32 (d,1,H_4), 8.44 (m, 2,$H_{3,5}$), 8.71 (d,1,H_2), 8.74 (s,1, H_6) and 8.85 ppm (AB,2,$H_{11,12}$); $J_{2,3} = J_{4,5} = 8.6$, $J_{7,8} = 11.2$, $J_{8,9} = J_{8,10} = 2.2$ and $J_{9,10} = 10.3$ Hz. 1-Nitro-BaP trans-9,10-dihydrodiol (acetone-d_6 with trace D_2O): 4.58 (dd,1,H_9), 5.79 (d,1,H_{10}), 6.46 (dd,1,H_8), 7.03 (d,1,

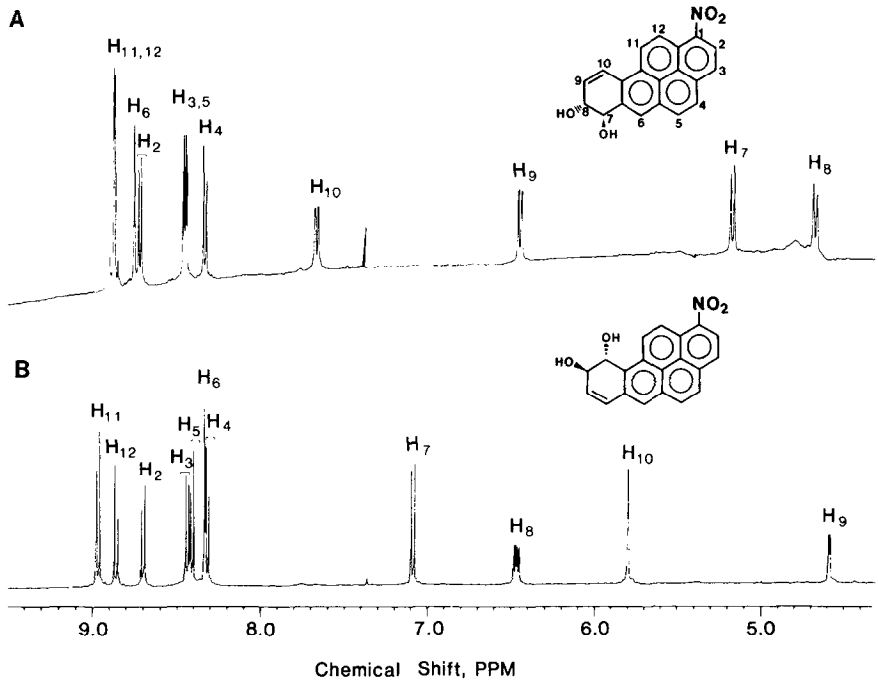

Fig. 4. 500 MHz proton NMR spectra of metabolites identified as (A) 1-nitro-BaP trans-7,8-dihydrodiol and (B) 1-nitro-BaP trans-7,8-dihydrodiol and (B) 1-nitro-BaP-trans-9,10-dihydrodiol. Chemical shifts are in ppm relative to tetramethylsilane.

H_7), 8.31 (d,1,H_4), 8.33 (s,1,H_6), 8.41 (d,1,H_5), 8.43 (d,1,H_3), 8.70 (d,1, H_2), 8.86 (d,1,H_{12}) and 8,96 ppm (d,1,H_{11}); $J_{2,3}$ = 8.6, $J_{4,5}$ = 9.0, $J_{7,8}$ = 9.5, $J_{8,9}$ = 5.6, $J_{9,10}$ = 2.2 and $J_{11,12}$ = 9.9 Hz.

For 1-nitro-BaP trans-7,8-dihydrodiol, the coupling constants between the carbinol protons ($J_{7,8}$ = 11.2 Hz) and between the non-benzylic olefinic and carbinol protons ($J_{8,9}$ = 2.2 Hz) clearly indicate that this trans-dihydrodiol preferentially adopts a quasidiequatorial conformation (20). However, for 1-nitro-BaP trans-9,10-dihydrodiol, the coupling constants between the carbinol protons ($J_{9,10}$ = 2.2 Hz) and between the non-benzylic olefinic and carbinol protons ($J_{8,9}$ = 5.6 Hz) indicate that this trans-dihydrodiol preferentially adopts a quasidiaxial conformation (21). Thus, the preference of the dihydrodiol conformation for the 1-nitro-BaP is the same as that of the respective BaP dihydrodiols (20,21).

The material contained in peak 1 had a uv-visible spectrum (Fig. 2B) that was similar to that of 7,8,9,10-tetrahydro-1-nitro-BaP, a synthetic standard.

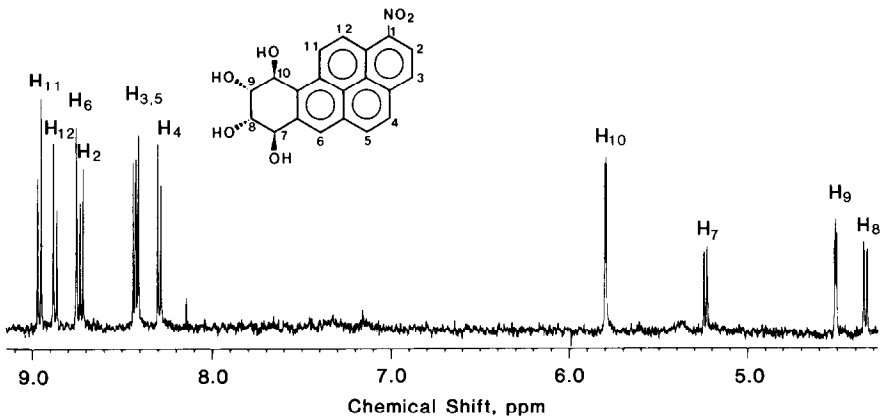

Fig. 5. 500 MHz proton NMR spectrum of metabolite identified as 1-nitro-BaP 7,8,9,10-tetrahydrotetrol.

This metabolite had a mass spectrum with molecular ions at m/z 365 and fragment ions at m/z 335 (loss of NO), 301 (loss of NO_2 and H_2O) and 299 (loss of NO and $2H_2O$). These data were consistent with the metabolite being a 1-nitro-BaP 7,8,9,10-tetrahydrotetrol. A high resolution 500 MHz proton NMR spectrum of this metabolite confirmed the structural assignment (Fig. 5). The proton NMR assignments are as follows: (acetone-d_6 with trace D_2O): 4.34 (dd,1,H_8), 4.51 (apparent t,1,H_9), 5.24 (d,1,H_7), 5.80 (d,1,H_{10}), 8.29 (d,1,H_4), 8.42 (m,2,$H_{3,5}$), 8.73 (d,1,H_2), 8.75 (s,1,H_6), 8.87 (d,1,H_{12}) and 8.96 ppm (d,1, H_{11}); $J_{2,3}$ = 8.6, $J_{4,5}$ = 9.0, $J_{7,8}$ = 9.0, $J_{8,9}$ = 2.2, $J_{9,10}$ = 3.4 and $J_{11,12}$ = 9.9 Hz. The observed coupling constants of $J_{7,8}$, $J_{8,9}$ and $J_{9,10}$ indicate that this tetrol preferentially adopts a half chair conformation and has a trans-cis-trans configuration between H_7-H_8, H_8-H_9 and H_9-H_{10}, respectively. To avoid steric interaction with the bay region hydrogen at C_{11} (22), the hydroxyl substituent at C_{10} maintains a quasidiaxial conformation. Consequently, the hydroxyl groups at C_7, C_8 and C_9 are at quasiequatorial, quasiequatorial and quasiaxial, respectively.

Although trans-dihydrodiols have been identified as metabolites of 1-nitropyrene (11), 6-nitrochrysene (23) and 7-nitrobenz[a]anthracene (24), it was not known if a vicinal dihydrodiol-epoxide could be enzymatically formed from metabolism of nitro-PAHs. In this report, we found 1-nitro-BaP is metabolized to a 7,8,9,10-tetrahydrotetrol which indicates that a vicinal dihydro-

diol-epoxide is a metabolite of 1-nitro-BaP. The trans-cis-trans configuration at the terminal benzo ring of the 1-nitro-BaP 7,8,9,10-tetrahydrotetrol metabolite implies that the metabolically formed vicinal dihydrodiol-epoxide is either 1-nitro-BaP trans-7,8-dihydrodiol anti-9,10-epoxide or 1-nitro-BaP trans-9,10-dihydrodiol anti-7,8-epoxide.

1-Nitro-BaP has been found to be a potent bacterial mutagen in Salmonella typhimurium in the presence of mammalian liver homogenate activation enzymes (13). In the tester strains TA98 and TA100, the mutagenic activation of 1-nitro-BaP is five-fold higher than that of BaP which itself is also a potent bacterial mutagen (13). These results together with our findings reported in this paper suggest that the enzymatically formed vicinal dihydrodiol-epoxide may be an ultimate mutagenic metabolite of 1-nitro-BaP when tested in the S. typhimurium with activation enzymes. On the other hand, 1-nitro-BaP is also a direct-acting bacterial mutagen while BaP is not (14). The direct-acting mutagenic activity of 1-nitro-BaP may be due to the nitro reduction of this compound to the activated form, N-hyroxy-1-amino-BaP by the nitroreductase(s) present in the bacteria (5). Thus, there appear to be at least two unique metabolic activation pathways for 1-nitro-BaP as far as its mutagenicity is concerned. The first is nitro-reduction to an N-hydroxy arylamine which is the activated metabolite of aromatic amines. The second is the ring oxidation to a vicinal dihydrodiol-epoxide which is a common ultimate mutagenic and carcinogenic metabolite of PAHs.

ACKNOWLEDGMENT

The authors are grateful to Dr. D.A. Miller for NMR spectral measurement, Dr. J.P. Freeman for mass spectral analysis, M. Moak for technical assistance and L. Amspaugh for typing this manuscript.

REFERENCES

1. Wang, Y.Y., Rappaport, S.M., Sawyer, R.F., Talcott, R.E. and Wei, E.T. (1978) Cancer Lett., 5, 39-47.
2. Lofroth, G., Hefner, E., Alfheim, I. and Moller, M. (1980) Science (Wash.), 209, 1037-1039.
3. Rosenkranz, H.S., McCoy, E.C. Sanders, D.R., Butler, M., Kiriazides, D.K. and Mermelstein, R. (180) Science (Wash.) 209, 1039-1043.
4. Schuetzle, D. (1983) Environ. Health Persp., 47, 65-80.
5. Rosenkranz, H.S. and Mermelstein, R. (1983) Mutat. Res., 114, 217-267.
6. El-Bayoumy, K., Hecht, S.S., and Hoffman, D. (1982) Cancer Lett., 16, 333-337.

7. Howard, P.C., Heflich, R.H., Evans, F.E., and Beland, F.A. (1983) Cancer Res., 43, 2052-2058.
8. McCoy, E.C., DeMarco, G., Rosenkranz, E.J., Anders, M., Rosenkranz, H.Sand Mermelstein, R. (1983) Environ. Mutagen, 5, 17-22.
9. Fu, P.P., Chou, M.W., Yang, S.K., Beland, F.A., Kadlubar, F.F. Casciano, D.A., Heflich, R.H. and Evans, F.E. (1982) Biochem. Biophys. Res. Commun. 105, 1037-1043.
10. Fu, P. P. and Chou, M.W. (1982) In: Cytochrome P-450, Biochemistry, Biophysics, and Environmental Implications, E. Hietanen, M. Laitinen and O. Hanninen (eds) Elsevier Biomedical Press, Amsterdam, The Netherlands, pp. 71-74.
11. El-Bayoumy, K., and Hecht, S.S. (1983) Cancer Res., 43, 3132-3137.
12. El-Bayoumy, K. and Hecht, S.S. (1982) Cancer Res., 42, 1243-1248.
13. Pitts, J.N., Jr., van Cauwenberghe, K.A., Grosjean, D., Schmid, J.P., Fitz, D.R., Belser, W.L., Jr., Knudson, G.B., and Hynds, P.M. (1978) Science 202, 515-519.
14. Pitts, J.N., Lokensgard, D.M., Harger, W., Fisher, T.S., Mejia, V., Schuler, J.J., Scorziell, G.M. and Katzenstein, Y.A. (1982) Mutation Res., 103, 241-249.
15. Kon, G.A.R. and Roe, E.M.F. (1945) J. Chem. Soc., 143-148.
16. Spitzer, U.A. and Stewart, R. (1974) J. Org. Chem., 39, 3936-3937.
17. Fu, P.P. and Harvey, R.G. (1978) Chem. Rev., 78, 317-361.
18. Chou, M.W. and Yang, S.K. (1979) J. Chromatogr. 185, 635-654.
19. Fu, P.P. and Harvey, R.G. (1977) Tetrahedron Lett., 2059-2062.
20. Jerina, D.M., Selander, H., Yagi, H., Wells, M.C., Davey, J.F., Mahadevan, V. and Gibson, D.T. (1976) J. Am. Chem. Soc., 98, 5988-5996.
21. Zacharias, D.E., Glusker, J. P., Fu, P.P. and Harvey, R.G. (1979) J. Am. Chem. Soc., 101, 4043-4051.
22. Yagi, H., Thakker, D.R., Hernandez, O., Koreeda, M., and Jerina, D.M. (1977) J. Am. Chem. Soc., 99, 1604-1611.
23. El-Bayoumy, K., Hecht, S.S. and Wynder, E.L. (1982) Proc. Am. Assoc. Cancer Res., 23, 84.
24 Fu, P.P. and Yang, S.K. (1983) Biochem. Biophys. Res. Commun., 115:123-129.